Measuring committed preadipocytes in human adipose tissue from severely obese patients by using adipocyte fatty acid binding protein

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Tchoukalova, Youinka D., Michael G. Sarr, and Michael D. Jensen. Measuring committed preadipocytes in human adipose tissue from severely obese patients by using adipocyte fatty acid binding protein. Am J Physiol Regul Integr Comp Physiol 287: R1132–R1140, 2004. First published July 29, 2004; doi:10.1152/ajpregu.00337.2004.—To understand the significance of the reported depot differences in preadipocyte dynamics, we developed a procedure to identify committed preadipocytes in the stromovascular fraction of fresh human adipose tissue. We documented that adipocyte fatty acid binding protein (aP2) is expressed in human preadipocyte clones capable of replication, indicating that it can be used as a marker of committed preadipocytes. Because aP2 expression can be induced in macrophages, stromovascular cells were also stained for the macrophage marker CD68. We found aP2+/CD68− cells (designated as committed preadipocytes) that did not have lipid droplets (true preadipocytes) and that did have lipid droplets <6.5 μm in diameter (very immature adipocytes). Adipose tissue from subcutaneous, omental, and mesenteric depots was obtained from nine patients undergoing bariatric surgery for measurement of stromovascular cell number, the number of committed preadipocytes (aP2+CD68−), aP2+ macrophages (aP2−CD68+), and aP2− macrophages (aP2−CD68−). The number of committed preadipocytes did not differ significantly between depots but varied >20-fold among individuals. Total cell number, stromovascular cell number, and the number of aP2− macrophages was less (P < 0.05) in subcutaneous than in omental fat (means ± SE, in millions: subcutaneous, 2.3 ± 0.3, 1.4 ± 0.3, and 0.17 ± 0.08; and omental, 4.8 ± 0.7, 3.8 ± 0.5, and 0.34 ± 0.06); mesenteric depot was intermediate. These data indicate that the cellular composition of adipose tissue varies between depots and between individuals. The ability to quantify committed preadipocytes in fresh adipose tissue should facilitate study of adipose tissue biology.

Preadipocytes; macrophages; fat distribution

Regional variations in fat mass accumulation are more predictive of metabolic abnormalities than is absolute fat mass per se (4, 6, 11, 30), but it is unclear why some individuals gain relatively more upper body fat, and especially, visceral fat. Understanding the mechanisms behind the differences in regional fat accumulation may provide a link between central obesity and insulin resistance underlying the metabolic syndrome. The expansion of adipose tissue mass depends on adipocyte hypertrophy up to a point beyond which hyperplasia becomes the limiting factor (29, 34). The fat cell number reflects the balance of preadipocyte proliferation, apoptosis, differentiation, and adipocyte apoptosis. Cultured preadipocytes from omental fat, obtained from people with severe obesity, have a reduced capacity for differentiation (20, 39) and proliferation (42) but are more susceptible to apoptosis (31, 33) than preadipocytes from subcutaneous abdominal fat. This implies a site-specific regulation of preadipocyte number.

A comprehensive interpretation of depot differences in preadipocyte dynamics is hindered by the lack of information regarding the number of committed preadipocytes in human adipose tissue. To understand whether depot differences in preadipocyte dynamics contribute to differences in accumulation of regional or whole body fat, it is important to determine how many preadipocytes are present. Culturing/ cloning techniques have been used in an attempt to measure the number of preadipocytes in adipose tissue, but the process of harvesting cells results in some (40–50%) loss of cell viability, which inevitably creates uncertainty as regards to how many preadipocytes were present originally (3). Thus we looked for an approach to identify committed preadipocytes that did not rely on the vagaries of cell viability.

The approach we investigated was to use a specific protein to distinguish preadipocytes from the other cell types present in the stromovascular (SV) fraction of adipose tissue. We tested whether adipocyte fatty acid-binding protein (aP2) could be a suitable marker. The mRNA of aP2 was originally identified by screening 3T3 adipocyte cDNA libraries for mRNAs that increase markedly during differentiation (5, 37). Small amounts of aP2 mRNA and aP2 protein were found in rodent preadipocyte cell lines and the adipose SV fraction when sensitive techniques were applied (14, 23, 25, 37). Likewise, aP2 mRNA was detected in cultured human preadipocytes in early stages of development (9). We examined whether committed human preadipocytes express the aP2 protein and found that it was present in cultured and subcultured preadipocytes, as well as preadipocyte clones that maintained their replicative potential. Because aP2 protein expression can be induced in macrophages (10, 16, 28), we used co-staining with the macrophage marker CD68 to distinguish aP2-positive cells in SV fraction of adipose tissue that were aP2+ macrophages and not preadipocytes. This dual immunofluorescent technique was used to identify committed preadipocytes in SV cells isolated from fresh adipose tissue. There was a wide (>20-fold) range in the number of committed preadipocytes per gram of adipose tissue. This variation in the number of committed preadipocytes suggests that the cellular composition is variable between depots and individuals.

Experimental Procedures

Human subjects and tissue handling. The experimental protocols were approved by Mayo Foundation Institutional Review Board. Nine
otherwise healthy subjects with severe obesity (body mass index 41–65 kg/m²; three males and six females; age range 33–53 yr) scheduled to undergo elective gastric bypass surgery gave informed written consent to participate in the study. Persons with acute illnesses, systemic inflammatory illnesses, treatment with corticosteroids, except nasally inhaled products, diabetes mellitus requiring insulin or oral agents, or malnutrition were excluded. Adipose tissue from subcutaneous, omental, and mesenteric depots (∼2 g) was excised during operation and handled immediately without prolonged warm ischemia. Three aliquots from each site (∼50 mg each) were put into separate tubes and flash frozen in liquid nitrogen or processed immediately for DNA analysis. About 50–100 mg tissue was saved for lipid extraction. The remaining tissue was transferred into 50-ml tubes for digestion. Surgical waste fat tissue from cosmetic liposuctions was obtained from four patients and used to validate aP2 protein expression in preadipocytes. Adipose tissue from liposuctions was washed with PBS and subjected to digestion.

**Lipid extraction.** Lipids were extracted by the method of Folch et al. (15) and the lipid content per gram of tissue calculated.

**Genomic DNA determination.** DNA was isolated using a Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, MN) following the manufacturer’s recommendations. Samples were kept at 4°C until the DNA measurement. Before the DNA measurement, each sample was mixed with the fluorescent nucleic acid stain PicoGreen dsDNA (Molecular Probes, Eugene, OR) as recommended by the manufacturer. Each sample was excited at 480 nm, and the intensity of fluorescence emission was measured at 560 nm with the use of a spectrofluorometer (model SLM 8000C; SLM-AMINCO, Urbana, IL). DNA concentrations were calculated using a standard curve of fluorescence from known concentrations of the bacteriophage λDNA in the range of 1 ng/ml-1 μg/ml. Duplicate measurements for each sample were obtained, and the DNA was calculated. Three separate samples were analyzed from each site, and the median value was chosen as a representative value for the DNA content per gram of tissue from that site.

**Tissue digestion.** Tissue was digested with collagenase type II (Sigma, St. Louis, MO) in 1 mg/ml HEPES buffer (0.1 M HEPES, 0.12 M NaCl, 0.05 M KCl, 0.005 M glucose, 1.5% wt/vol BSA, 1 mM CaCl₂, 2 mM H₂O₂, pH 7.4) for 15 min. This cell suspension was centrifuged for 5 min at 300 g at room temperature (RT).

About 150 μl of the resulting floating layer containing fat cells were used for measurement of fat cell size. Early collection of fat cells was done to avoid fat cell breakage. The cell pellet of the SV fraction and the remainder of the floating layer were resuspended back in the same collagenase-HEPES buffer and incubated for another 15–20 min to accomplish more thorough digestion. The cell suspension was then filtered through a 250-μm nylon mesh and then centrifuged for 10 min at 300 g at RT. The resulting cell pellet was resuspended and incubated in ethyroycicyte lysis buffer (0.154 M NH₄Cl, 0.01 M KHCO₃, and 0.1 mM tetrasodium EDTA, pH 7.3) for 10 min at RT. These SV cells were centrifuged for another 10 min at 300 g at RT. Cell pellets were resuspended in 1 ml of cold PBS, counted, adjusted to a concentration of 1 × 10⁵ cells/ml, and 200 μl of the cell suspension were deposited on slides (2 × 10⁴ cells/slide) with the use of Cytospin 3 Cell Preparation System (Thermo Shandon, Pittsburgh, PA) at 600 rpm and medium acceleration for 6 min. The cytospin cells were used for immunocytochemistry. Cell pellets from fat tissue obtained from liposuctions were either lysed for immunoblotting or cultured.

**Fat cell sizing: mean fat cell lipid content determination.** Fat cell sizing was carried out as we described previously (40). The mean fat cell lipid content and adipose tissue lipid content were used to calculate fat cell number (see Calculations). Preadipocyte cultures were initiated from the SV fraction in 10% fetal bovine serum as described by Tchkonia et al. (39). Some confluent 3-day cultures were switched to serum-free adipogenic medium. Preadipocytes from days 3, 7, and 15 from these cultures were either immunostained for αP2 or lysed for immunoblotting. Other confluent 3-day cultures were subcultured by trypsinizing and repating in medium with 10% FBS at a density of 3 × 10⁴ cells/cm² until confluence. Preadipocytes were repeatedly subcultured in this way. Preadipocytes from the tenth subcultures and preadipoctyes that had been subcultured 28 times (a kind gift from Dr. James Kirkland, Boston University Medical Center, Boston, MA) were cytospun as aforementioned and immunostained for αP2.

**Preadipocyte cloning.** Preadipocyte cloning was performed as described before (39). After 2 wk, colonies were evident, and by 3 wk, some colonies were confluent. Preadipocyte clones were then stained for αP2 or αP2 plus lipid.

**Immunofluorescence.** For the dual αP2-CD68 immunostaining, cytospun SV cells from subcutaneous, omental, and mesenteric depots were thawed, rinsed with PBS, and fixed with cold acetone for 1 min and then dried for 10 min. Cells were rinsed and incubated in DAKO protein-blocking buffer (DPBB) containing 0.1% saponin (DPBB/SAP) for 10 min. Rabbit polyclonal antibody against murine recombinant αP2 that has been affinity purified (a kind gift from Dr. David A. Bernlohr, Department of Biochemistry, University of Minnesota, Minneapolis, MN; 100 dilution) and saponin (to a final concentration of 0.1%) were added to a ready-to-use mouse anti-human CD68 (KPI) antibody (DAKO Cytomation, Carpinteria, CA), and 50 μl of the mixture was applied to each slide for 1 h at RT in humidifying cassettes. After three washes, cells were incubated in a mixture of 200-diluted secondary antibodies AlexaFluor 680 goat-anti-mouse and AlexaFluor 488 chicken anti-rabbit in DPBB/SAP (both from Molecular Probes, Eugene, OR) for 30 min at RT in the dark. Cells were washed and incubated in 5 μg/ml solution of Hoechst-33258 (bisBenzimide; Sigma, St. Louis, MO) for 10 min at RT in the dark. Cells were washed and mounted with ProLong Antifade kit (Molecular Probes). Negative controls using a mixture of universal negative controls, mouse and rabbit (both from DAKOCytomation), in place of the specific primary antibodies, were run in parallel with each positive slide from each depot.

For the other staining procedures, the similar scheme was followed with minor modifications. For example, for the αP2 and lipid staining, the CD 68 antibody and its respective secondary antibody were omitted, and a stock solution (1 mg/ml in DMSO of Nile red (Molecular Probes) was added to the Hoechst-33258 in 1:1,000 dilution.

For the αP2, CD68, and lipid staining, the secondary antibodies were conjugated with AlexaFluor 350 goat anti-mouse and AlexaFluor 680 goat anti-rabbit, followed by incubation with Nile red without Hoechst-33258.

For the αP2, CD68, and CD14 staining, cytospun cells from the SV fraction were cultured preadipocytes maintained in 10% FBS for three days were incubated in a mixture of mouse anti-human CD14 antibody (BD Biosciences, San Jose, CA), diluted to 0.75 μg/ml, plus antibody against αP2 for 1 h. After being washed, the cells were incubated in a mixture of AlexaFluor 568 goat anti-mouse IgG and AlexaFluor 680 goat anti-rabbit (both from Molecular Probes) for 30 min. A second blocking/permeabilization step using DPBB/SAP was performed, followed by incubation with monoclonal CD68 antibody conjugated with FITC (DAKO Cytomation) for 20 min. Parallel staining with controls was performed with an additional step controlling for CD68/FITC antibody with FITC-conjugated rat anti-mouse IgG1 (BD Biosciences; ×50 dilution).

Images of fluorescently labeled cells were collected immediately on a confocal microscope (model LSM510, Zeiss, Oberkochen, Germany) equipped with an Axiovert 100M inverted microscope and a LD-Achroplan ×40/0.6 numerical aperture objective. Green fluorescence from AlexaFluor 488 fluorochrome-conjugated antibodies or Nile red was excited with the 488-nm line of an argon/krypton laser. Emission was collected through a 505–550 nm band-pass filter. Red fluorescence from AlexaFluor 680 fluorochrome-conjugated antibodies was excited with a 633 nm helium/neon laser. Emission was...
collected through a 650-nm long-pass filter. Blue fluorescence from the Hoechst dye or Alexa Fluor 350-conjugated antibody was excited with an argon laser at 364 nm. Emission was collected through a 385- to 470-nm band-pass filter.

Single-stained cells, aP2*CD68* (green) and aP2*CD68+ (red), and dual-stained cells, aP2*CD68* (green and red), were counted by hand from at least 1,000 cells determined by nuclei (blue) and counted with image analysis software (KS400, Zeiss). Thus the percentage of aP2*CD68*, aP2*CD68+, and aP2*CD68+ cells was determined and used in the calculations for the number of preadipocytes, aP2–macrophages, and aP2+macrophages, respectively. The dual aP2–lipid staining resulted in images containing aP2–lipid+ (red, green), aP2+lipid– cells (red), and aP2–lipid– cells (blue nuclei).

The diameters of the biggest lipid droplets in cells with multiple lipid droplets were measured using the KS400 image analysis software. The images from the triple staining were screened for aP2–CD68+–lipid– cells.

Immunoblotting. The SV cell pellet obtained after digestion was reconstituted in lysis buffer (60 mM Tris base, pH 6.8, 1% SDS, and 5% glycerol). At the designated times, the preadipocyte cultures were rinsed and then lysed by the addition of the reconstituted in lysis buffer (60 mM Tris base, pH 6.8, 1% SDS, and 5% glycerol). The aP2 protein expression was determined by densitometry with an enhanced chemiluminescent plus detection system and Hyperfilm (both from Amersham Life Sciences, Piscataway, NJ). The results of Western blot analysis were scanned, and the aP2 protein expression was increased with time.

To assess whether aP2 could be used to identify committed preadipocytes in human adipose tissue, we addressed three issues. First, what is the time course of aP2 expression during adipogenesis (can it distinguish “early” preadipocytes)? Second, is aP2 only present in SV cells that have already begun to accumulate lipid? Third, do macrophages in the adipose tissue SV fraction express aP2 in a manner that is distinguishable from preadipocytes?

To address the first question, we used both preadipocyte culture and preadipocyte cloning techniques. The SV fraction of adipose tissue from fresh human fat tissue obtained as surgical waste from liposuction was plated. Because endothe-

RESULTS

Assessment of aP2 as preadipocyte marker. To assess whether aP2 could be used to identify committed preadipocytes in human adipose tissue, we addressed three issues. First, what is the time course of aP2 expression during adipogenesis (can it distinguish “early” preadipocytes)? Second, is aP2 only present in SV cells that have already begun to accumulate lipid? Third, do macrophages in the adipose tissue SV fraction express aP2 in a manner that is distinguishable from preadipocytes?

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<tr>
<th>Protein Density of aP2 Relative to Actin</th>
<th>3d</th>
<th>7d</th>
<th>15d</th>
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<td></td>
<td>10</td>
<td>12</td>
<td>16</td>
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Fig. 1. A: immunoblots of total cell lysates from preadipocyte cultures maintained in growth medium, day 3 (3d), are positive for adipocyte fatty acid binding protein (aP2). Switch to adipogenic medium in preadipocyte cultures increases markedly aP2 protein levels. Bottom, density of aP2 protein expression relative to β-actin confirms the marked increase of aP2 protein expression during preadipocyte differentiation. B: immunoblots of total cell lysates from stromovascular (SV) cells isolated by collagenase digestion of human subcutaneous adipose tissue are positive for aP2 protein.
express aP2. Thus in human tissue the expression of aP2 protein occurs very early during human preadipocyte commitment.

To be more confident that we were looking specifically at preadipocytes (adipocyte lineage cells still capable of replication), we used the cloning technique to grow preadipocytes from single cells. The immunofluorescent staining is shown in Fig. 2A. All of the cloned preadipocytes were positive for aP2. Cells were stained for lipid with Nile red; distinct lipid droplets were not present, but faint homogenous staining of whole cells was seen (data not presented). Thus proliferating preadipocytes (from clones) express aP2 before lipid droplet formation. From this study, we concluded that aP2 is a sufficiently early marker of preadipocyte development that would be a useful tool, provided it could be detected in the SV fraction of fresh adipose tissue and we could satisfactorily address the issue of macrophage expression of the protein.

To address the second and third questions, we performed immunoblotting and immunofluorescent staining of the cell pellet of the SV fraction from fresh adipose. Immunoblotting showed expression of aP2 (Fig. 1B). Dual immunofluorescent staining for aP2 and the macrophage marker CD68 (Fig. 3B) revealed numerous single-stained cells for aP2 alone (aP2⁺CD68⁻) cells, representing cells from the adipocyte lineage (Fig. 3A). Dual staining for aP2 and lipid droplets using Nile red demonstrated cells that stained positively for aP2 but that did not contain lipid droplets (aP2⁺lipid⁻) (Fig. 3B). Some of the aP2 positive cells contained very small (up to 6.5 μm) lipid droplets (Fig. 3C). To test whether cells without lipid droplets were all macrophages, we triple-stained cells for aP2, CD68, and lipid droplets (data not shown). Cells staining positively for aP2 but not for lipid droplets or CD68 (aP2⁺CD68⁻lipid⁻ cells) were plentiful. Taken together with the data obtained by studying mass cultures of preadipocytes and preadipocyte clones, we concluded that positive aP2 immunofluorescent staining in the absence of positive CD68 staining identifies committed preadipocytes and very immature fat cells.

Numerous CD68⁺ cells were also present. The majority of them were single-stained (aP2⁻CD68⁺) cells. There is some evidence that under certain conditions murine preadipocytes acquire phagocytic and microbicidal activities similar to macrophages and may express certain macrophage cell markers (12). If this is common in humans, it is possible that CD68⁺ cells we observed were preadipocytes. To analyze whether the CD68-positive cells in the SV fraction were largely preadipocytes, we stained cytospin preadipocytes precultured in 10% FBS for 3 days with antibodies against aP2, CD68, and another macrophage marker CD14, a receptor for endotoxin (Fig. 4F). All aP2⁻CD68⁺ cells also stained positive for CD14 and morphologically were larger than the aP2⁺CD68⁻CD14⁻ cells. Both features are characteristic of macrophages, and,
Fig. 3. Fluorescent confocal microscopy of cytopunstromovascular cells isolated after collagenase digestion of human subcutaneous adipose tissue. A: dual immunostaining for aP2 (Alexa 488, green fluorescence) and CD68 (Alexa 680, red fluorescence), and counterstaining of nuclei with Hoechst-33258 (blue fluorescence) shows single-stained cells (arrowheads) and dual-stained cells (arrow); magnification ×40. B: dual staining for aP2 (Alexa 680, red fluorescence) and lipids (Nile red, green fluorescence), and counterstaining of nuclei with Hoechst-33258 (blue fluorescence) demonstrates aP2-positive cells with no lipid accumulation (arrows); magnification ×100. C: another area from B staining shows aP2-positive cells with various degree of lipid accumulation (arrows).

Fig. 4. Triple immunostaining for aP2 (Alexa 680, light blue fluorescence), CD68 (FITC, green fluorescence), CD14 (Alexa 568, red fluorescence), and counterstaining of nuclei with Hoechst-33258 (dark blue fluorescence); magnification ×40. A–E: images from cytopunstromovascular cells isolated after collagenase digestion of human subcutaneous adipose tissue (A–D, split images, E, overlay) showing aP2^−CD68^−CD14^− cells (blue arrows), an aP2^−CD68^−CD14^− cell (oval), aP2^−CD68^−CD14^− cells (white arrow), and a gigantic aP2^−CD68^−CD14^− cell with three nuclei (circle). F: an image from cultured preadipocytes in 10% FBS for 3 days demonstrating aP2^−CD68^−CD14^− cells (blue arrow) and aP2^−CD68^−CD14^− cells (white arrow).
hence, suggested to us that macrophages contaminate this 3-day cultured SV fraction of human adipose tissue. The staining of SV cells with antibodies against aP2, CD68, and CD14 resulted co-staining of nearly all aP2−CD68+ with CD14 (Fig. 4, A–E, arrow, circle). Only a few cells were aP2−CD68−CD14− (Fig. 4, A–E, oval). A small fraction of CD68+ cells (Fig. 3A, arrow) or CD68+CD14+ cells were aP2 positive. Most of these cells were mononuclear, but some were large and multinucleated (Fig. 4, A–E, circle). Collectively, this data suggested that, with the dual staining for aP2 and CD68, macrophages comprised of two (aP2− and aP2+) subfractions and that the aP2+ macrophages are not preadipocytes.

Application of aP2 as preadipocyte marker. The means ± SE of the adipose tissue measurements we performed are shown in Table 1. The total cell number per gram of adipose tissue in subcutaneous depot was less than in omental fat (P < 0.01). The total cell number per gram mesenteric fat was intermediate and did not differ significantly from the other two depots. The lipid content per gram of tissue was highest in subcutaneous, less in omental, and least in mesenteric depots. The mean lipid content per fat cell, although not different statistically, followed the profile of the lipid content per gram of tissue between depots. Thus the fat cell number per gram of adipose tissue was not different between depots. The SV cell number per gram of tissue (the difference between total cell and fat cell numbers) was greater in omental fat than in subcutaneous fat (P < 0.01). There were no differences between tissue depots in the proportion of committed preadipocytes and aP2− macrophages as assessed by the aP2 and CD68 staining of SV cells. However, the aP2− macrophages (aP2−CD68+ cells) comprised a higher proportion of SV cells in subcutaneous fat compared with omental and mesenteric depots (subcutaneous vs. omental, P = 0.06; subcutaneous vs. mesenteric; P < 0.02); there was no difference in the percentage of aP2− macrophages between the two visceral depots. The number of preadipocytes per gram of adipose tissue showed large interindividual variations but no consistent inter depot differences (Tables 1 and 2). The number of aP2− macrophages was greater in omental compared with the other subcutaneous and mesenteric depot (P = 0.02). However, the number of aP2+ macrophages per gram of tissue did not differ between depots.

### DISCUSSION

People differ with respect to the tendency to expand their fat mass, both in total body fat mass and body fat distribution. Differences in total or regional preadipocyte characteristics may play a role in this variability. To date, estimating preadipocyte number has been done by cell culture techniques (3, 43). Culturing live SV cells from digested adipose tissue samples can be done in such a manner as to stimulate preadipocytes, which are otherwise not morphologically distinguishable, to become mature adipocytes. This differentiation is proof that the parent cell was capable of becoming a fat cell, the working definition of a preadipocyte. However, to calculate preadipocyte number using cell culture technology, one must know what portion of cells, and specifically preadipocytes, lose viability in the process. Our goal was to identify cells in fresh human adipose tissue that are committed to the preadipocyte pathway in a manner that is not confounded by variations in cell viability. We found that aP2 is expressed early in the differentiation of human preadipocytes. This observation suggests that it is a suitable marker to identify committed preadipocytes in fresh human adipose tissue, although a portion of the cells could be classified as very immature adipocytes. A significant proportion (5–10%) of SV cells stained positive for aP2 but not for CD68, i.e., were not macrophages. This approach should provide an index of the regulation of preadipocyte dynamics that will compliment preadipocyte culture techniques.

The substantial abundance of aP2 protein and mRNA in rodent adipocytes and the marked increase in expression during

### Table 1. Selected measurements and derived cell numbers per gram of adipose tissue by depots

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<th>Subcutaneous</th>
<th>Omental</th>
<th>Mesenteric</th>
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<tr>
<td>Total cell number, million</td>
<td>2.2 ± 0.3*</td>
<td>4.8 ± 0.7†</td>
<td>3.5 ± 0.8†</td>
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<td>Lipid content/g tissue, g</td>
<td>0.74 ± 0.01*</td>
<td>0.70 ± 0.02‡</td>
<td>0.64 ± 0.02‡</td>
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<td>Lipid content/fat cell, µg</td>
<td>0.90 ± 0.09</td>
<td>0.84 ± 0.11</td>
<td>0.78 ± 0.13</td>
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<tr>
<td>Number of fat cells, million</td>
<td>0.9 ± 0.1</td>
<td>1.0 ± 0.3</td>
<td>1.0 ± 0.1</td>
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<tr>
<td>Stromovascular cell number, million</td>
<td>1.4 ± 0.3*</td>
<td>3.8 ± 0.5†</td>
<td>2.5 ± 0.8†</td>
</tr>
<tr>
<td>Fraction of stromovascular cells as preadipocytes, %</td>
<td>10 ± 3</td>
<td>6 ± 1</td>
<td>6 ± 1</td>
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<tr>
<td>Fraction of stromovascular cells as aP2− macrophages, %</td>
<td>9 ± 3</td>
<td>9 ± 1</td>
<td>7 ± 1</td>
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<td>Fraction of stromovascular cells as aP2− macrophages, %</td>
<td>5 ± 1*</td>
<td>2 ± 1*</td>
<td>1 ± 0.4†</td>
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<tr>
<td>Number of preadipocytes, thousand</td>
<td>180 ± 79</td>
<td>220 ± 52</td>
<td>185 ± 84</td>
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<td>Number of aP2− macrophages, thousand</td>
<td>157 ± 88*</td>
<td>317 ± 64†</td>
<td>245 ± 112‡</td>
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<td>Number of aP2+ macrophages, thousand</td>
<td>67 ± 27</td>
<td>73 ± 26</td>
<td>41 ± 20</td>
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Values are expressed as means ± SE. AP2, adipocyte fatty acid binding protein. *‡†P < 0.05, means within a row that do not share a common superscript differ.
prechadipocyte differentiation (18, 41) has contributed to the image of aP2 as a late marker of differentiation. We found that dividing human preadipocytes express aP2 protein (detected in preadipocyte subcultures and in preadipocyte clones and their subcultures) before exposure to adipogenic medium. This observation confirms that aP2 is an early marker for committed human preadipocytes. If aP2 were present only in terminally differentiated cells, it would be less useful in estimating the number of preadipocytes in fresh human adipose tissue. Although aP2 protein was not detected by immunoblotting lysates from undifferentiated, cultured human subcutaneous preadipocytes in one study (39), differences in the methodology likely explain the discrepancy. In the present study, we used ~10-fold the amount of total cellular proteins and the more sensitive ECLplus detection kit. Thus we were likely able to show earlier expression of aP2 protein than has been appreciated. In the blots of the differentiating cultures, aP2 protein presented itself as two isoforms. It has been shown that the unphosphorylated form of aP2 shows high binding affinity for fatty acids (8), which explains functionally their increment during differentiation. However, the role of the phosphorylated form of aP2 that was predominantly observed in the growing cultures has not been yet clarified. What is known about the regulation of aP2 transcription that might support early activation of the adipogenic pathway? In murine preadipocytes, aP2 gene transcription is mediated by the AP-1 transcription factor complex (2, 13, 24, 35, 36, 46). Various transcription factors differentially modulate the nature and quantity of proteins comprising the AP-1 transcription factor complex (2, 13, 24, 35, 36, 46). It is possible that the AP-1 transcription factor complex mediates the early aP2 gene expression in human preadipocytes as well. Furthermore, peroxisome proliferator-activated receptor-γ (PPAR-γ) may modulate the early aP2 gene expression: we detected weak PPAR-γ signals in preadipocyte clones by immunocytochemistry and in lysates from preadipocyte cultures harvested after 3 days treatment with FBS by immunoblotting (data not shown). Identifying the nature of the components of AP-1 complex in human preadipocytes, given its role in murine aP2 gene regulation, and clarification of the role of PPAR-γ in the early aP2 protein expression warrants further investigation.

Because we found no direct methods for measuring the relative proportion of preadipocytes, we used an indirect approach. The total cell number was determined by measuring DNA content per gram of adipose tissue. Measuring DNA is a good measure of total cell number due to the invariability of cellular DNA content during even extreme physiological conditions (17) and the excellent correlation with the cell number (26). One previous study (32) reported DNA concentration in human subcutaneous fat tissue as a substitute for total cellularity, but the number of cells calculated from these data was greater than in our study (2.4 vs. 5.8 million). Differences in the body mass index of the participants in these two studies (26 vs. 55 kg/m2) likely account for this discrepancy because more small adipocytes are to be expected per gram of tissue in lean adults. To the best of our knowledge, our study appears to be the first report of total and SV cell number in intra-abdominal adipose tissue. Whether the greater cellularity of omentum fat seen in our population (severe obesity) will be found in nonobese adults needs to be assessed. Two studies that measured cell number in the isolated SV fraction from subcutaneous fat by measurement of DNA concentration (32) or cell counting (43) report ~2 and 0.14 million SV cells per gram of tissue, respectively. Our finding of ~1.35 million SV cells per gram of subcutaneous fat tissue is close to the results of O’Brien et al. (32).

In contrast to the differences in total cell number and the number of SV cells per gram of adipose tissue, we found a similar number of fat cells in each of the three depots that we examined. From a perspective of (pre)adipocyte dynamics, if the fat cell mass/size and number are constant, then the number of fat cells dying, presumably via apoptosis, should equal those maturing. The literature provides evidence for between-depot differences in these processes. For example, omental preadipocytes are more predisposed to apoptosis than subcutaneous preadipocytes (31, 33). A greater percentage of cultured subcutaneous preadipocytes than omental preadipocytes differentiate into mature adipocytes (20, 39). If these observations reflect the in vivo situation, these data imply that subcutaneous fat can preferentially add new mature fat cells. Other data suggest that apoptosis increases as preadipocytes from both depots differentiate into adipocytes (33), however, so that the “advantage” of subcutaneous fat may be lost at this point in the fat cell life cycle. The rate of apoptosis of terminally differentiated subcutaneous and omental adipocytes from these two depots is not different (A. Sorisky, unpublished observations). How and whether these differences in the rates of differentiation and apoptosis of subcutaneous versus intra-abdominal preadipocytes contribute to the appearance of new adipocytes is unknown. If more subcutaneous adipocytes appear, this observation would imply that the turnover rate (e.g., apoptosis) of subcutaneous adipocytes is greater than omental adipocytes. If it were possible to combine information regarding the number of committed preadipocytes as well as to assess apoptosis in mature fat cells, this approach would help to determine the in vivo relevance of findings from cultured preadipocytes. The widely varying number of committed preadipocytes we found contrasts with the narrow range of fat cell number suggesting that there are interindividual and/or depot differences in the rate of maturation of preadipocytes, that the mature adipocytes have different life spans, or that the rate of apoptosis of preadipocytes varies widely.

The SV fraction of adipose tissue contains a heterogeneous population of cells (22, 47). We documented that CD68-positive cells stain macrophages. As best we can determine, this is the first study to quantify the number of macrophages in human adipose tissue from different depots. It is interesting that we found more aP2+ macrophages in omental fat than subcutaneous fat in these severely obese patients. A greater number of macrophages in adipose tissue of murine models of obesity have been reported (21, 45). This process was more apparent in intra-abdominal depots than in the subcutaneous depot in mice (21). The presence of macrophages correlates with increasing weight and fat cell size and occurred primarily by recruitment of monocytes from the circulation in mice (44). The technique we describe may be applicable to studies of cells in addition to committed preadipocytes.

Some of the assumptions we used and potential limitations of this work must be acknowledged. First, to extrapolate from the percent committed preadipocytes and SV cell number to total preadipocyte number, one makes the implicit assumption that the SV fraction we collected is representative of the entire
SV fraction, i.e., that digestion does not selectively release some kinds of cells into the SV fraction and not others. We harvested SV cells after 40–45 min of digestion to minimize the time between tissue sampling and cell collection; our concern was that more prolonged exposure of cells to digestion conditions might alter the expression of proteins from those present in vivo (for example, activation of macrophages leading to expression of ap2). A longer collagenase digestion may increase the absolute recovery of adipose SV cells, but maximal recovery was not pursued as stromovascular number was defined indirectly (the difference between total cell number and the number of fat cells per gram of tissue) and we have no reason to suspect that a shorter digestion time would selectively release only some types of cells. Second, although we use the term “committed preadipocyte” in this report, we know that ap2-positive cells in the SV fraction include both committed preadipocytes and very immature fat cells (i.e., small lipid droplets present). Thus the ap2−/CD68+ cells we observed really were a combination of both cell types. If it were possible to quadruple stain (nuclear stain, ap2, CD68, and Nile red), we could distinguish committed preadipocytes from very immature adipocytes, but this is not yet a routine option at our facility. Third, this study does not define how early ap2 protein is expressed during preadipocyte development. There may be adipose tissue SV cells with a capacity to differentiate into adipocytes that are too undifferentiated to express ap2 [ap2−/−] preadipocytes, which could be characterized as preadipocyte precursors or “adipoblasts.” At present this population cannot be readily defined due to lack of specific markers for early preadipocytes and adipoblasts. Gronthos et al. (19) detected CD105 (endoglin) and CD166 (activated leukocyte cell-adhesion molecule) in adipose-derived stromal vascular cultures. These markers have been used to define a stromal cell population from bone marrow with the potential to differentiate into adipocytes, chondrocytes, or osteoclasts, defined as mesenchymal stem cells (27). The specificity of these proteins for the adipose-derived stem cells needs further validation. Furthermore, we do not know whether the population of adipose-derived stem cells consists of one type “common stem cells” or is heterogeneous. Human Dlk1, equivalent to the rodent Pref-1, expressed in murine preadipocytes (38), could be a candidate for a marker of early preadipocytes. Overexpression of Dlk1/Pref-1 in bipotential human mesenchymal stem cell line created from bone marrow aspirates prevents commitment of these cells to either osteocyte or adipocyte cell lineages (1), suggesting a role of Dlk1/Pref-1 in these cells. However, data on the expression of Dlk1/Pref-1 in human adipose-derived stromal cells are lacking. Therefore, at present, the approach of using ap2 protein expression should provide a feasible and useful way to assess how many SV cells are entering an important phase of adipocyte development.

In summary, we report that the protein ap2 is present in early human preadipocytes, making it an acceptable marker for these otherwise indistinguishable cells in fresh human adipose tissue. Immunofluorescent staining allowed us to determine the number of committed preadipocytes/very immature adipocytes and ap2-expressing macrophages in subcutaneous, omental and mesenteric fat of patients with severe obesity. About 8% of SV cells were committed to the adipocyte lineage, but there was a >20-fold range of committed preadipocytes per gram of adipose tissue, suggesting marked variation in this characteristic.

Fat cell number was much less variable, tending to be similar and independent of depot. Combining measures of committed preadipocyte number and characteristics of the preadipocyte dynamics using cell culture approaches offers the opportunity to better understand the biology of adipose tissue.

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